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NOTE

Resemblance to the Enemy's Eyes Underlies the Intimidating Effect of Eyespots

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ABSTRACT: Eyespots of some prey are known to deter predators, but the reason for this response has not yet been established, and thus the taxonomically widespread occurrence of this color pattern has remained an evolutionary conundrum. Two alternative hypotheses propose that (1) the eyelike appearance of the pattern falsely indicates the presence of the predator's own enemy or (2) predators are hardwired to be cautious toward conspicuous prey. Earlier research has pertained mainly to eyespots in butterflies. Here we tested the hypothesis that eyespots resemble eyes by utilizing the lateral position of eyes in fishes. This allowed us to produce eyelike displays that did not have the round appearance of eyespots. Our study indicates that eye mimicry is an important factor evoking hesitation in predators. Moreover, we present direct evidence that this is because predators associate eyelike displays with the threat posed by their own enemies.

Keywords: eye mimicry, eyespot, deception, intimidation, predation, protective coloration.

Introduction

Eyespots are marks that consist of concentric rings of contrasting colors and have received their name because they resemble, more or less, the vertebrate eye (Poulton 1890; Cott 1940; Blest 1957). Eyespots have been studied particularly in Lepidoptera (Blest 1957; Brakefield et al. 1996; Lyytinen et al. 2004; Vallin et al. 2005; Kodandaramaiah et al. 2009; Olofsson et al. 2012b, 2013), but they are also found in many other taxa, such as mollusks, fishes, amphibians, reptiles, and birds. Due to their taxonomically widespread occurrence, their adaptive function has intrigued evolutionary biologists for over a century (Poulton 1890). In different species, eyespots may have

different functions. For example, in some cases, they are involved in reproductive signaling (Robertson and Monteiro 2005; Gagliano and Depczynski 2013). Some eyespots have a divertive (deflective) function, such that they manipulate attacking predators to aim their strikes in a direction that facilitates prey escape or to a body part where only minor damage is likely (Vallin et al. 2011; Kjernsmo and Merilaita 2013; Olofsson et al. 2013; Prudic et al. 2015; Kjernsmo et al. 2016). However, predator deterrence is the effect that is perhaps best known. Such eyespots, known as “intimidating eyespots,” increase the chance of prey escape by making the predator hesitate or halt its attack (Cott 1940; Blest 1957; Vallin et al. 2005; Stevens et al. 2007; Kodandaramaiah et al. 2009; Merilaita et al. 2011; Hossie and Sherratt 2013).

The first rigorous evidence for the deterring effect has been presented only recently (Vallin et al. 2005), but the idea is not new (Poulton 1890; Cott 1940; Blest 1957), and the reason why some eyespots deter predators is still under debate (Stevens et al. 2007, 2008, 2009; Janzen et al. 2010; Merilaita et al. 2011; Blut et al. 2012; Hossie and Sherratt 2013; Stevens and Ruxton 2014; De Bona et al. 2015). According to the eye mimicry hypothesis, eyespots are a deceptive signal, such that the eyelike marks give the predator a false indication of the presence of its own enemy. While many researchers have found this idea appealing (e.g., Janzen et al. 2010), the problem thus far has been in demonstrating that it is the eyelike appearance and not some other property of the pattern that deters predators. Furthermore, the eye mimicry hypothesis assumes a mechanistic chain that involves a predator experiencing a threat posed by its enemy and then associating that threat with its prey, due to a visual similarity between the prey (eyespot) and the enemy (eye). This mechanistic chain still lacks evidence.

To humans, many eyespots bear a resemblance to the eye, but due to taxon-specific differences in visual perception and interpretation of visual information, this may not be how predators perceive these marks. Therefore, Blest (1957) suggested an alternative explanation for the deterring effect of intimidating eyespots. According to the conspicuous signal

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hypothesis, it is the conspicuousness of the eyespot pattern, not their eyelike appearance, that deters predators. Although the adaptive benefit of such a response may not be apparent, it is known that sudden bright displays of prey coloration (i.e., deimatic displays) can startle predators (Olofsson et al. 2012a). Also, the eyespot pattern may be particularly salient and promote sensory overload or induce neophobia in inexperienced predators (Stevens 2005; Stevens and Ruxton 2014). There is some experimental evidence suggesting that conspicuousness of eyespots due to contrast, size, or number may deter predators (Stevens et al. 2007, 2008, 2009).

To address the long-outstanding questions about the deterring effect of eyespots, we conducted an experiment with two aims. Our first aim was to test the validity of the assumption about the associative chain underlying the eye mimicry hypothesis. According to this assumption, a focal predator that is exposed to predation threat from its own enemy forms an association between the threat and a visual feature of the enemy, the eye. A prey should thus be able to evoke the association and startle the focal predator by imitating this feature.

Our second aim was to compare the importance of eye-like appearance and conspicuousness for the deterring effect of eyespots. If eye mimicry underlies the deterring effect of eyespots, then not only displays that resemble the typical, circular shape of eyespots but also displays resembling other projections of the eye (resulting in shapes unlike the circular shape of eyespots) should deter predators. Therefore, we also needed displays that resembled eyes but not eyespots. Many predators, such as birds of prey or mammalian carnivores, have their eyes roughly on a plane that is perpendicular to the anterior-posterior axis of their body, such that a prey approached by the predator faces the eyes. In a frontal view of these predators, the shape of the eyes corresponds to the typical shape of eyespots. This is not the case for many other predators, such as predatory fish, which have eyes on the sides of the head. From a lateral view of the fish, the shape of the eye corresponds to the typical shape of eyespots. However, from a frontal view, the eyes instead consist of the laterally protruding spherical lens and the pupil with the shape of a dorsoventrally elongated ellipse and, thus, are distinct from the typical eyespot pattern. Importantly, because of the broad angle of view of fishes' eyes, both lateral and frontal projections of a predatory fish's eye should represent a threat to its prey. Consequently, the use of fish provides a valuable opportunity to investigate the underlying mechanism for the deterring effect of eyelike displays and the function of eyespots. Here, we used threespine sticklebacks (*Gasterosteus aculeatus*) as a model for fish cognition and behavior. We studied their responses toward noneyelike and eyelike patterns based on the appearance of the eyes of their natural predator, the perch (*Perca fluviatilis*), and how these responses depend on previous experiences of predation threat.

Methods

Study Species

The parental generation of the threespine sticklebacks used as the focal predators and the perch used as their enemy were collected in early June 2012 from the same area of the Baltic Sea in southwestern Finland. Perch were used because they are a common predatory fish in the Baltic Sea and also a main fish predator of sticklebacks in the area where we collected the fish (e.g., Gross 1978; Mustamäki et al. 2014; Byström et al. 2015). Several aspects make the threespine stickleback an ideal species for this study. It is primarily a visual predator (Wootton 1976; Ohguchi 1978; Litvak and Leggett 1992; Hart and Gill 1994; Rowe et al. 2004), and it responds to visual stimuli and attacks artificial prey items (Kjernsmo and Merilaita 2013; Kjernsmo et al. 2016). Although wild sticklebacks may often encounter the eyes of their predators, eyespots are uncommon in their prey, and so there is no reason to believe that sticklebacks have an evolutionary history of preying on animals with eyespots. This means that our experimental system is able to address the broad evolutionary question of whether animals, in general, are predisposed to avoid prey with eyespots should they encounter them.

We used the wild-caught sticklebacks to generate a laboratory population through artificial fertilization. This ensured that the sticklebacks that entered our experiment were predator naive and also parasite free. Five weeks after the laboratory generation had hatched, the fish were assigned to a treatment that lasted over 6 months and involved cues of predation threat (fig. A1; figs. A1, A2 available online). During this treatment, half of the juvenile sticklebacks were exposed recurrently to predation threat in the form of visual and olfactory cues of live perch, chemical alarm cues from conspecifics, and visual cues of predation (perch feeding on pieces of sacrificed adult sticklebacks). Olfactory cues were included because threespine sticklebacks are known to respond to them (Frommen et al. 2011). For the control, the other half of the juveniles were treated otherwise identically, except that they did not receive any visual predator cues and received water instead of the chemical cues. Full details about the collection of fish, their rearing conditions, and the long-term treatment are presented in the appendix, available online.

Setup

We used a three-trophic setup to test the deterring effect of eyespots. The focal predator was the threespine stickleback, which preyed on artificial prey items. Perch served as the predator stimulus, which allowed us to create predator-exposed and predator-naïve test populations. To test the importance of eye mimicry and the conspicuousness of prey signal for the deterring effect, we presented the sticklebacks

with four different prey types that had one of the following four patterns: (1) no additional mark (fig. 1A); (2) a noneyelike, rectangular mark (fig. 1B); (3) a single eyelike mark (equal in area to the rectangular mark) based on the lateral view of a perch's eye and resembling a typical circular and concentric eyespot pattern (fig. 1C); and (4) an eyelike pattern (consisting of two eyelike marks) based on the frontal view of a perch's eyes, which differed from the typical eyespot pattern (fig. 1D). Each prey item contrasted with the background to an equal extent. According to our hypothesis, if eye mimicry underlies the deterring effect of eyespots, then eyelike displays, independently of whether they are eyespot-like or not, should elicit a stronger response than the two noneyelike displays. If, on the other hand, conspicuousness of the signal underlies the deterring effect of eyespots, then we would expect that equally conspicuous prey signals, independent of whether they are eyelike, would elicit an equally strong response.

Before presentation of prey items to the sticklebacks, we had assigned the fish to the long-term treatment that involved cues of predation threat. This treatment enabled our second aim, to test for the plausibility of the chain of associations that is implied by the eye mimicry hypothesis. In other words, we assumed that, if the predator associates the prey display with the appearance of and threat imposed by its enemy, then the predator-experienced sticklebacks should be more hesitant than predator-naïve sticklebacks to attack prey that have displays that resemble predator eyes.

Predation Experiment

We tested the behavioral response of the sticklebacks toward the different prey patterns (fig. 1) in a predation ex-

periment. The prey items were made of a rectangular piece of water-resistant paper (31 mm × 11 mm) with a piece of red mosquito larvae (*Chironomidae* spp.) placed on top as the edible component. The prey items had black patterning printed on white paper. All four prey types had a mottled background pattern. To produce the two eyelike patterns, we anesthetized 11 perches (length: 9.5–15.0 cm) then photographed them to produce binary images of lateral and frontal views of eyes (see appendix for details of the prey).

The sticklebacks' response toward the prey patterns was tested from February to April 2013. For these tests, we used six identical 30-L aquaria that were lit from above by fluorescent lamps that simulate natural light. For each replicate, one stickleback was carefully placed into the start zone (SZ; fig. A2) of an experimental aquarium. Two frozen chironomid larvae were then placed on the gray foraging plate (FP; fig. A2) located in the foraging zone (FZ) on top of a prey item containing no additional marking (fig. 1A). The divider separating the SZ from the rest of the tank was then lifted, allowing the fish to locate and feed on the chironomids in the FZ and to acclimatize to the experimental aquaria overnight. On the following day, the day of the experiment, each fish was gently moved back into the SZ before the observations started. A prey item, randomly chosen among the four prey types (fig. 1), was placed on the FP. A chironomid larva was placed on the prey item, and 2 min later, the divider was lifted and the observation started. Each fish had 15 min to locate and attack the prey item. We recorded the latency to attack (defined as the time it took for a stickleback to attack the prey item after initiating approach; i.e., when the fish was above the FP with its head down toward the prey). We replicated each of the eight treatment groups 15 times, equaling 120 individuals tested in total. Each fish was used only once.

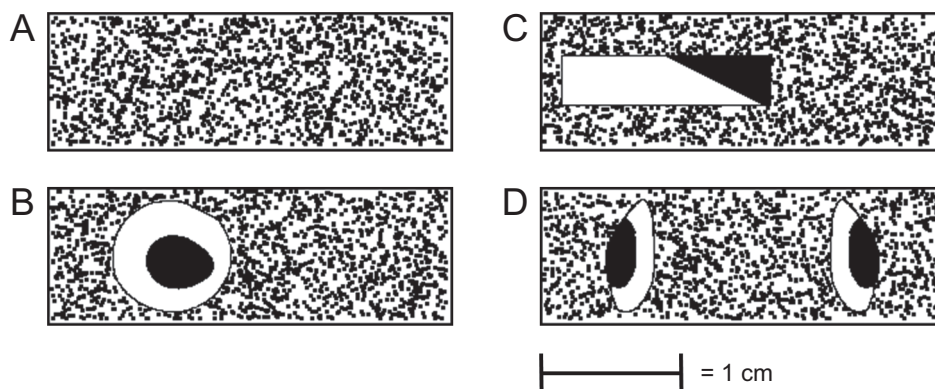


Figure 1: Four different prey types used in the behavioral experiment. The two noneyelike controls had either no additional mark (A) or a noneyelike rectangular mark (B). The two prey types with eyelike marks had either one mark reproduced from the lateral view of a perch's eye and resembled the typical eyespot pattern (i.e., concentric circles; C) or two eyelike marks reproduced from the frontal view of a perch's eyes (D). The ratios of black to white were equal for all prey types.

The data from the experiment are deposited in the Dryad Digital Repository, <http://dx.doi.org/10.5061/dryad.43kp7> (Kjernsmo and Merilaita 2017).

Statistical Methods

The dependent variable, attack latency, was \log_{10} transformed for normal distribution and homoscedasticity. We first conducted a two-way ANOVA with planned, orthogonal contrasts to test for the effects of prey patterning, predator experience, and their interaction on attack latency. To complete the analysis, we compared the prey that had the two eyelike patterns (fig. 1C, 1D) with the prey that had the rectangular, noneyelike mark (fig. 1B) using two-tailed, two-sample t -tests for which the α values were corrected using the sequential Bonferroni correction. All analyses were conducted using R for Windows (ver. 2.9.2; R Development Core Team 2009).

Results

Attack latency of the sticklebacks was significantly influenced by an interaction between the predation treatment and prey pattern ($F_{3,112} = 3.82$, $P = .012$) and by prey pattern alone ($F_{3,112} = 25.88$, $P < .0001$; fig. 2, table A1, available online). The fish took longer to attack the two prey types that exhibited eyelike marks than to attack the two prey types without eyelike marks ($t_{112} = 8.69$, $P < .0001$; fig. 2). This clearly shows that it is the likeness to a vertebrate eye that underlies the deterring effect of prey marks. Since

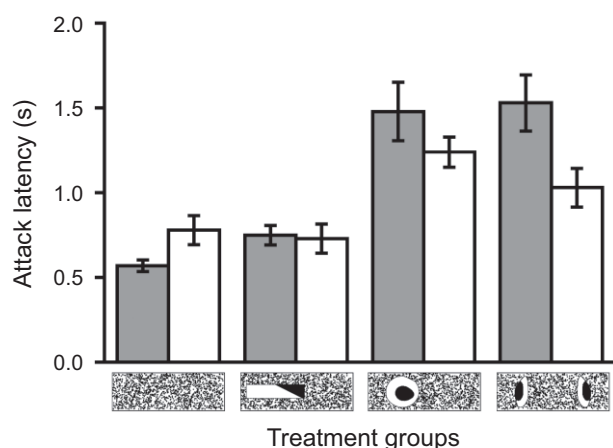


Figure 2: Mean (\pm SE) attack latencies in seconds for the four types of prey (from left to right: no additional mark, a noneyelike rectangular mark, a single eyelike mark corresponding to lateral view of the eye, and two eyelike marks corresponding to frontal view of the eyes) and two preexperimental treatment groups, predator experienced (gray bars; the sticklebacks had been subjected to visual and olfactory cues of predation) and predator naive (white bars); $n = 15$ for each of the eight groups.

the eyelike mark and the rectangle had equal areas of black and white, our experiment does not support the hypothesis that it is conspicuousness that causes the deterring effect.

Previous experience with predation cues markedly increased the hesitation induced by the two eyelike patterns in the sticklebacks ($t_{56} = 2.81$, $P = .0058$; fig. 2). In contrast, there was no effect of such experience on their response toward the two noneyelike patterns ($t_{56} = 1.00$, $P = .32$; fig. 2). This suggests that the sticklebacks associated the eyelike appearance of marks with previous experiences of predators. Attack latency did not differ significantly between the two eyelike patterns in the predator-experienced fish ($t_{28} = 0.11$, $P = .91$) and the predator-naive fish ($t_{28} = 1.69$, $P = .093$). Neither did it differ significantly between the two noneyelike prey types, without a mark and with a rectangle, in predator-experienced ($t_{28} = 1.76$, $P = .081$) and predator-naive ($t_{28} = 0.50$, $P = .62$) fish. These two latter comparisons suggest that the use of the control prey without a mark in the acclimatization of the fish did not bias the response of the fish noticeably.

Finally, we specifically compared the fish's response toward the equally conspicuous, noneyelike rectangular mark and both the eyelike displays. The predator-experienced fish took a significantly longer time to attack prey with the single eyelike mark than to attack those with the rectangular mark ($t_{28} = 4.03$, $P < .001$, $\alpha_{\text{corrected}} = 0.0125$; fig. 2). Similarly, the predator-experienced fish also took more time to attack the prey with two eyelike marks than to attack those with a noneyelike mark ($t_{28} = 4.72$, $P < .001$, $\alpha_{\text{corrected}} = 0.0167$; fig. 2). Interestingly, attack latency was also longer for both of the eyelike patterns than it was for the noneyelike mark among the fish that had no experience of predation cues (two eyes: $t_{28} = 4.20$, $P < .001$, $\alpha_{\text{corrected}} = 0.025$; single eye: $t_{28} = 2.23$, $P = .034$, $\alpha_{\text{corrected}} = 0.05$; fig. 2). Thus, the sticklebacks were more hesitant to attack prey items that had eyelike patterns reproduced from their natural predator's eyes than to attack those with an equally conspicuous but noneyelike pattern.

Discussion

We used the threespine stickleback to demonstrate how eye mimicry in combination with a predator's previous experience of cues of its own enemies can buy the prey valuable time to escape predation. Although more studies are needed to confirm the generality of this protective mechanism involving three trophic levels, we believe that it may account for the deterring effect of eyespots in a broad range of taxa and environments.

We found that the sticklebacks that had been subjected to predation cues were more hesitant to attack the prey items than were the predator-naive sticklebacks, but only if the prey items sported eyelike marks. This supports an associa-

tion between the threatening experience imposed by the enemy of a predator and the increased aversion of the predator toward prey that mimic a feature of the predator's enemy. Thus, the results show the plausibility of eye mimicry as an explanation of the deterring effect of eyespots.

The sticklebacks were more hesitant to attack prey items that displayed either of the eyelike marks than to attack prey items that displayed no mark or a noneyelike mark. This indicates that it is the eyelike appearance rather than some other property, such as the conspicuousness of the signal, that is the essential characteristic of the prey signal.

At first glance, one might find the differences in effect in our experiment small when considering prey protection. For example, the display corresponding to the frontal view of two eyes increased the attack latency, on average, for 0.95 s compared with the attack latency induced by the control. Considering the burst swimming speed of 131 cm/s of threespine sticklebacks (Garenc et al. 1999), this would correspond to an additional head start of 125 cm for the escaping prey of a stickleback. Generally, in fish, ambush or lunging predators rarely pursue their prey far beyond its location at the start of its flight, particularly if the prey has gained a head start. Therefore, most fish predator pursuits and prey escapes are relatively short, often less than 2 s (Godin 1997). For these reasons, we find it reasonable to assume that even a brief hesitation can give a critical advantage to the prey, although the outcome of each encounter depends on the circumstances and the species involved.

The mechanism underlying the deterring effect of eyespots and the importance of eyelike appearance therein has been a long-standing question in research on protective coloration. Our results contradict earlier studies that suggest that the conspicuousness of eyespots causes their deterring effect (Blest 1957; Stevens et al. 2008, 2009; Stevens and Ruxton 2014). Some recent studies have used field experiments in which predation on edible rewards of artificial prey was checked with intervals of 1 h or longer (Stevens et al. 2008, 2009). As this study and other results (Kodandaramaiah et al. 2009; Merilaita et al. 2011) show, the hesitation caused by intimidating eyespots in an attacking predator wears off in seconds, at least when the display is stationary. Therefore, infrequent observation of predation does not appear to be methodologically ideal. Hence, the effect of contrast may be explained by reasons other than deterrence (e.g., contrasting marks distracting the attention of untrained predators away from the reward; Stevens et al. 2008; Dimitrova et al. 2009).

Considering signal evolution, some degree of conspicuousness is necessary for transmittance of visual signals, but there is no clear hypothesis for why conspicuousness as such would be a property that gives useful information about threats and thus could act as an adaptive deception. Eyes, on the other hand, have a high degree of similarity, at least

across all vertebrate taxa, when related to the variability in general morphology of vertebrates, and therefore it is easy to see how eyelike shapes can act as useful visual cues of the presence of another animal. This also suggests that concealment of the eye can be important for prey camouflage (Kjernsmo et al. 2016) and camouflage of predators (this study).

Albeit not manipulating predators' experience of their enemy directly, some earlier studies have presented some indirect evidence that predators might associate eyespots with the threat posed by their own enemies. For example, Olofsson et al. (2012b) showed that domestic fowl exposed to butterflies with intact eyespots were more vigilant and elicited more alarm calls typically associated with ground predators than those that were exposed to butterflies that had their eyespots painted over. Furthermore, other recent experiments have suggested that, rather than their conspicuousness, features that make the displays more eyelike are central to the deterring effect of eyespots (Merilaita et al. 2011; Blut et al. 2012).

In this study, even the predator-naïve sticklebacks showed some hesitation when they attacked the prey that had eyelike patterns, but the response was strengthened markedly by earlier experience with predators. This suggests that the response toward eyelike markings was partly innate and partly acquired through experience. Because the enhanced hesitation caused by experience of predation cues was observed only when the sticklebacks received prey that had eyelike patterns, this result also clearly supports the significance of eye mimicry.

Considering that both eyelike displays in our experiment caused hesitation in attacking fish, it is interesting that eyespots in aquatic prey, such as fish, are circular (Kelley et al. 2013). We think that developmental mechanisms of color patterns may provide a possible explanation: concentric circles are relatively simple to produce (Beldade and Brakefield 2002; Dilão and Sainhas 2004), and for this reason, they may be more common than other eyelike shapes.

An interesting question is what determines the protective efficacy of intimidating eyespots. In a recent experiment on divertive eyespots, subsequent presentations weakened the diversion, implying that habituation can influence the effect (Kjernsmo and Merilaita 2013). However, due to the severity of the threat underlying eye mimicry, we find the deterring effect less prone to wear off. In Batesian mimicry, the ratio of the mimics to the models is a crucial determinant of the protective effect (Fisher 1930; Pfennig et al. 2001). Similarly, we would anticipate that the protective effect of eye mimicry depends on at least two factors. One is the frequency of false enemy eyes that the predators encounter. Another factor is the intensity of the risk imposed on the predators by their own enemies. The evolutionary lability of eyespots found in some fishes (Kelley et al. 2013) might reflect changes in selection on eyespots because of these factors.

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A threespine stickleback hesitantly approaches a prey item that has eyelike displays. Photo credit: Karin Kjærnsmo.